Studying Elite Suppressors to Understand Mechanisms of Immune Control of HIV

by

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ABSTRACT

Individuals who control HIV infection despite not being treated with antiretroviral therapeutics, deemed Elite Controllers or Suppressors (ES), represent a model for a functional cure. MHC class I alleles HLA-B*57 and HLA-B*27 have been associated with the ES phenotype, suggesting a genetic component. Accordingly, the role of CD8+ T cells in the ability to control infection is an ongoing field of investigation. ES have qualitatively greater CTL responses to HIV infection in *in vitro* and *ex vivo* assays. HIV often escapes from CTL pressure by mutating epitopes that are presented by MHC molecules. These CTL escape mutations can prevent CD8-mediated killing if the infected host cannot recognize these modified viral epitopes. Interestingly, escape variants are often found in the plasma of ES but not in integrated HIV-proviral genomes. In this thesis, I will present evidence that ES can suppress viral replication in CD4+ T cells that are infected with HIV variants harboring CTL escape mutations.

While ES have qualitatively more effective CD8+ T cells *ex vivo*, this does not preclude other possible factors from being critical to the phenotype. The frequency of latently infected CD4+ T cells in the periphery of ESs is significantly lower than in chronically infected individuals; this phenotype is not due to an inability for the CD4+ T cells to be infected. The amount of intracellular RNA in infected cell types has not been well characterized. We hypothesized that ongoing viral replication due to a lack of antiretroviral therapy may lead to increased baseline cell associated HIV-1 mRNA levels, which may allow for greater CTL-mediated clearance of HIV, in turn keeping the size of the HIV-infected CD4 population low. Here we characterize baseline and induced expression of cell-associated HIV-1 mRNA levels in CD4+ T cells from ES.

PhD Dissertation Referees for Christopher W Pohlmeyer

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In the existence that I experience, the double pendulum only has one starting condition. I am where I am by living the life I have lived, which is the only life I could have known. It is therefore of no surprise that things are how they are. And every situation in every corner of this existence has been critical in my arriving at my current circumstance, yes. No single step in my life was sufficient for placing me here today, yet every one was necessary. Nevertheless, my perception of fate is colored by the human condition; specific instances appear to have greater degrees of significance than others. While my thanks is ultimately senseless, the sentiment remains and can be appreciated to contain value.

Let's do this chronologically.

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INTRODUCTION

ES are HIV-infected individuals who control viremia without antiretroviral therapy[1,2,3,4]. Due to the very low level of viremia, these individuals almost never, if ever, transmit virus to a new host. Additionally, ES rarely lose control and progress to AIDS. Understanding the mechanism by which these individuals are able to control HIV infection could lead to the discovery of a functional cure for HIV. Early studies suggested that these individuals were merely infected with a defective HIV variant[5,6]. While infection with replication deficient viral strains may lower the burden to control HIV infection, many ES are infected with fully replication competent strains[7,8,9,10].

Nevertheless, it is entirely possible that control is merely happenstance. ES may be the individuals who reside in one tail of a normal distribution centered around some median viral load[11]. While this idea is difficult to disprove, there does exist, to some degree, a hierarchy of HLA alleles with respect to the ability to control viremia[12,13]; this suggests that immune pressure is involved in control of viremia. Also, ES disproportionately possess HLA-B*57 and HLA-B*27 alleles compared to the general population, both seronegative and seropositive[14,15,16,17,18,19,20]. This association of HLA molecules with control of viremia strongly suggests a genetic component to the ES phenotype, specifically a CD8-mediated mechanism of control.

Possessing protective HLA alleles is not sufficient for immune-mediated HIV control. Upon stimulation with HIV peptides, HIV-specific CD8+ T cells isolated from the periphery proliferate and generate cytokines; peptide stimulation of CD8+ T cells incurs a more robust phenotype in ESs than MHC-matched individuals who do not control[18,21] (Chronic Progressors, or CPs). Additionally, coculturing CD8+ T cells isolated from the blood of ES with HIV-infected autologous CD4+ T cells results in greater inhibition of viral production than observed in CP counterparts[22].

HIV evades this CTL pressure *in vivo* by generating mutations in MHC-restricted epitopes. These CTL escape mutations are either not processed, loaded, or recognized properly, and the ternary complex cannot form. CD4+ T cells from ES generally harbor few CTL escape mutations, whereas CPs often harbor several[23]. Interestingly, escape variants can be found in the plasma of ES, and this does not necessarily lead to loss of control[24]. In chapter 1, we will characterize the ability for CD8+ T cells to respond to stimulation with either wild type or escape variant HIV Gag peptides and suppress viral infection in CD4+ T cells infected with HIV variants that either do or do not contain CTL escape mutations.

Without antiretroviral therapy, the low level of plasma viremia observed in ES may be a result of ongoing replication. Antiretroviral therapy is believed to halt viral replication[25,26]. Without ongoing replication, antigen cannot be presented to CD8+ T cells, and CTL responses will dissipate[27,28]. If ES experience ongoing replication, CD8+ T cells will be continually primed for infection, improving the cytotoxic capacity of CD8+ T cells in a way that may not be translatable to CPs. It is poorly understood what the baseline levels of cell-associated HIV mRNA are and if these levels are due to ongoing replication. In chapter 2, we characterize cell-associated HIV mRNA in CD4+ T cells of ES.

CHAPTER 1: CD8+ T CELLS FROM HLA-B*57 ELITE SUPPRESSORS EFFECTIVELY SUPPRESS REPLICATION OF HIV-1 ESCAPE MUTANTS.

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ABSTRACT

Background

Elite Controllers or Suppressors (ES) are HIV-1 positive individuals who maintain plasma viral loads below the limit of detection of standard clinical assays without antiretroviral therapy. Multiple lines of evidence suggest that the control of viral replication in these patients is due to a strong and specific cytotoxic T lymphocyte (CTL) response. The ability of CD8+ T cells to control HIV-1 replication is believed to be impaired by the development of escape mutations. Surprisingly, viruses amplified from the plasma of ES have been shown to contain multiple escape mutations, and it is not clear how immunologic control is maintained in the face of virologic escape.

Results

We investigated the effect of escape mutations within HLA*B-57-restricted Gag epitopes on the CD8+ T cell mediated suppression of HIV-1 replication. Using site directed mutagenesis, we constructed six NL4-3 based viruses with canonical escape mutations in one to three HLA*B-57-restricted Gag epitopes. Interestingly, similar levels of CTL-mediated suppression of replication in autologous primary CD4+ T cells were observed for all of the escape mutants. Intracellular cytokine staining was performed in order to determine the mechanisms involved in the suppression of the escape variants. While low baseline CD8+ T cells responses to wild type and escape variant peptides were seen, stimulation of PBMC with either wild type or escape variant peptides resulted in increased IFN-γ and perforin expression.

Conclusions

These data presented demonstrate that CD8+ T cells from ES are capable of suppressing replication of virus harboring escape mutations in HLA-B*57-restricted Gag epitopes. Additionally, our data suggest that ES CD8+ T cells are capable of generating effective de novo responses to escape mutants.

BACKGROUND

In primary HIV-1 infection, vigorous viral replication results in plasma virus levels as high as one million copies/mL of HIV-1 RNA. As cellular immune responses develop, plasma virus levels decrease, and a viral set point is established. A subset of HIV-1-infected individuals known as Elite Controllers or Suppressors (ES), maintain a viral set point below the limit of detection of standard clinical assays (<50 copies of HIV-1 RNA/mL of blood[1,2,3,4]). Early studies suggested that some long-term non-progressors (LTNPs) and ES are infected with defective viruses[5,6]. In contrast, more recent studies have determined that many ES are infected with viruses that are fully replication competent, suggesting that host factors rather than infection by defective virus are responsible for ES status[7,8,9,10,29].

Some HLA alleles affect disease progression, including HLA-B*27, HLA-B*51, HLA-B*57/58, and HLA-B*35[12,30]. Multiple cohort studies have demonstrated that the HLA-B*57 allele is overrepresented in ES[14,15,16,17,18,19,20]. This finding has been confirmed by multiple GWAS studies[31,32,33,34,35,36]. However, the majority of HIV-1-infected HLA-B*57$^+$ patients develop progressive disease, and are thus termed chronic progressors (CPs)[14]. The protection conferred by HLA-B*57 and HLA-B*27 is thought to be mediated by effective CD8$^+$ T cell responses against conserved immunodomniant epitopes that are presented by MHC class I proteins[18,21,22,37,38,39,40]. Comparison studies of HLA-B*57$^+$ ES and CPs have provided some details about the elite suppressor phenotype. Stimulation of bulk peripheral blood mononuclear cells (PBMCs) with Gag peptides

induces greater proliferation as well as more robust granzymes A/B and perforin expression in $CD8^+$ T cells from HLA-B*57$^+$ ES compared to HLA-B*57$^+$ CPs[18].

Some nonsynonymous mutations in epitopes enable the virus to escape from the cytotoxic T lymphocyte (CTL) responses. The role these escape mutations play in determining protection versus progression in HLA-B*57 positive patients is controversial. A correlation between the number of HLA-B*57 Gag epitopes and the level of viremia was observed in a cohort of HLA-B*5703 positive patients with Clade C HIV-1 infection[41]. In another study, the development of escape mutations was temporally associated with virologic breakthrough in a patient who had maintained undetectable viral loads for a year after infection[34]. In contrast, studies of early infection in other HLA-B*57/5801 positive patients have not found a correlation between the accumulation of escape mutations and virologic breakthrough[43,44]. Furthermore, one study found no difference in the frequency of escape mutations in HLA-B*57-restricted epitopes in proviral clones amplified from ES when compared to CPs[45]; other studies found that while escape mutations were largely absent from provirus amplified from $CD4^+$ T cells of ES, virus amplified from the plasma of the same subjects contained a high frequency of escape mutations[24,46]. In this study, we sought to explain how ES maintain undetectable levels of plasma virus despite the presence of circulating HIV-1 isolates that contain numerous escape mutations. Specifically, we asked whether $CD8^+$ T cells from these patients were capable of inhibiting the viral replication of engineered escape mutants. We demonstrate that ES can inhibit the replication of escape variant HIV-1 and suggest that these patients are capable of generating protective *de novo* responses against the escape mutant variants. This work has implications for the design of therapeutic T cell vaccines to prevent the progression of HIV-1 disease.

RESULTS

Effect of escape mutations in HLA-B*57-restricted Gag epitopes on viral fitness

The effect of several escape mutations on viral fitness has been explored *in vivo* and *in vitro*[47,48,49,50,51,52]. We focused on escape mutations in the three HLA-B*5703 restricted Gag epitopes: IW9 (Gag 147–155), KF11 (Gag 162–172), and TW10 (Gag 240–249). We have previously demonstrated that HLA-B*5703 positive ES in our cohort do not target the fourth HLA-B*57-restricted Gag epitope QW9 (Gag 308–316)[24] and therefore we did not include this epitope in our analysis. To address the fitness cost of these escape mutations, we introduced a series of mutations into the reference isolate NL4-3 and generated GFP-expressing HIV-1 pseudoviruses carrying these mutations. Virus concentrations were measured in triplicates by RT-PCR. We then infected PHA-activated $CD4^+$ T cells from four seronegative healthy donors using a 2 log range of virus inoculums. Infection curves for the seven viruses are shown in Figure 1A.

Infectivity relative to the reference clone NL4-3 was taken as a measure of fitness. Infectivity curves plateaued at different points for different mutants. A best fitting curve was generated with GraphPad to calculate a nonlinear least squares regression model and was used to determine the theoretical maximal infection. Figure S1 shows the best fit curve for each of the assayed viruses. This theoretical maximal infection was used to determine the relative percentage of maximal infection relative to the reference clone NL4-3 (Figure 1B). The I147L and A146P mutations, which are found in the IW9 epitope of p24, each had a minimal effect on viral fitness with calculated fitness levels of 88 percent and 90 percent respectively compared to unmutated NL4-3. The A163S mutation (72 percent relative fitness) had a larger effect on fitness consistent with a prior study that showed that mutations in this epitope in Clade C virus had a major impact on replication capacity[41]. When the A163S mutation was combined with the A146P mutation, a further decrease in fitness was observed (50 percent relative fitness). The

T242N/G248A variant had an intermediate level of fitness (67 percent relative fitness). Interestingly, the A146P/A163S/T242N/G248A variant (49 percent relative fitness) showed no additional loss of fitness than what was observed with the A146P/A163S variant.

ES CD8$^+$T cells suppress replication of viruses containing CTL escape mutations

We next determined whether CD8$^+$ T cells from ES could inhibit the infection of viruses harboring escape mutations. We isolated CD4$^+$ and CD8$^+$ T cells from seven HLA-B*5703$^+$ ES and five healthy seronegative donors. The ES did not have any other protective alleles. Unstimulated CD4$^+$ T cells were infected with each variant individually and were co-cultured with unstimulated CD8$^+$ T cells at various effector to target ratios for three or five days. Interestingly, unstimulated CD8$^+$ T cells from ES were capable of suppressing each variant (Figure 2A), whereas healthy donors had no suppressive capabilities (Figure 2D). The percentage of suppression decreased at lower effector to target ratios. Compared to day three, day five showed increased levels of suppression at all effector to target ratios (Figure 2A).

A prior study demonstrated that CD8$^+$ T cells that were stimulated with peptides were more effective at eliminating HIV-1 infected target cells[18]. We therefore stimulated PBMCs from the same group of ES and healthy donors with a combination of three wild type peptides (wild type IW9, wild type KF11, and wild type TW10) or a combination of three escape variant peptides (mutant IW9 (I147L), mutant KF11 (A163S), or mutant TW10 (T242N/G248A)) in the presence of 10 units/mL IL-2. After seven days of stimulation, CD8$^+$ T cells from both stimulation groups were individually isolated and cultured with unstimulated CD4$^+$ T cells that were infected with either wild type virus or one of the 6 escape mutants. Interestingly, there was no statistical difference in the suppressive capacity of CD8$^+$ T cells that were stimulated with either wild type or escape

variant peptides (Figure 2B, C and Figure S2). Figure 3 shows the suppressive ability of unstimulated $CD8^+$ T cells from each patient at day three and day five. Statistically significant differences in levels of suppression between wild type and escape variants were seen only for A163P at a one to two effector to target ratio (Figure 3A); this difference was not seen at any other effector to target ratios. Taken together, these results demonstrate that ES are capable of recognizing escape variant epitopes as effectively as their non-mutated counterparts.

Stimulation of $CD8^+$T cells with wild type or escape variant peptides increases expression of interferon γ and perforin

To determine the mechanism of CTL suppression of escape mutants, we analyzed the expression of IFN-γ and perforin in freshly isolated $CD8^+$ T cells and $CD8^+$ T cells that were primed with either wild type or escape variant peptides. Freshly isolated PBMCs and PBMCs from each stimulation group were stimulated overnight with individual wild type or escape variant peptides. Freshly isolated $CD8^+$ T cells were observed to have very low levels of IFN-γ expression in response to each peptide (Figure 4A, B, C left panel). In contrast, culture of PBMCs with wild type peptides over a 7 day period prior to overnight stimulation with wild type peptides resulted in a statistically significant increase ($P < 0.05$) in IFN-γ expression (Figure 4A, B, C center panel). A similar response was seen when the $CD8^+$ T cells were stimulated with the analogous escape variant peptide. Interestingly, IFN-γ expression in response to restimulation with wild type or escape variant peptides individually were similar in $CD8^+$ T cells that were cultured for 7 days in the presence of either wild type or escape variant peptides (Figure 4A, B, C right panel).

Because perforin expression is associated with CTL-mediated killing[18,39,53], we also examined perforin expression by stimulated $CD8^+$ T cells. Low levels of perforin and IFN-γ double positive cells were observed when freshly isolated $CD8^+$ T cells were

stimulated with wild type or escape variant peptides (Figure 4D, E, F left panel). In contrast, $CD8^+$ T cells that had been cultured for 7 days in the presence of wild type or escape variant peptide cocktails were observed to have an increase in the percentage of IFN-γ and perforin double positive $CD8^+$ T cells when stimulated overnight with either wild type or escape variant peptides. Interestingly, culturing PBMCs in the presence of wild type peptides or escape variant peptide cocktails resulted in similar levels of $CD8^+$ T cells that co-expressed IFN-γ and perforin in response to overnight peptide stimulation. Additionally, overnight stimulation with KK10 (Gag 263–272), an immunodominant HLA-B*27 specific peptide, resulted in no increase in IFN-γ or perforin expression, confirming the specificity of the enhanced $CD8^+$ T cell responses to the HLA-B*57-restricted peptides.

To determine whether similar responses were present in HLA-B*57 positive CPs, we also analyzed $CD8^+$ T cells from five CPs on suppressive HAART regimens, two CPs who recently had detectable viremia, and one CP with high levels of viremia who was not on HAART. With freshly isolated PBMCs, there was very low IFN-γ expression on $CD8^+$ T cells in response to overnight stimulation with peptides (Figure 5A, B, C left panel). Stimulation of cells with wild type peptides or escape variant peptides induced an increase in IFN-γ expression by $CD8^+$ T cells in some patients after overnight stimulation with TW10 or KF11 (Figure 5A, B, C center and right panels). The percentage of $CD8^+$ T cells that co-expressed IFN-γ and perforin increased from the unprimed baseline (Figure 5E, F, G), though generally not as dramatically as the increase seen in ES. This is consistent with the enhanced proliferative capacity of ES HIV-specific $CD8^+$ T cells[18,21]. Interestingly, the patients on HAART who recently had detectable levels of viremia had higher responses than the patients on suppressive HART regimens who maintained undetectable viral loads consistent with a boosting effect of viral replication.

DISCUSSION

CTL responses against Gag epitopes have been associated with virologic control[54,55,56] and Gag-specific $CD8^+$ T cells can target incoming virions and therefore have the potential to kill cells prior to productive infection[57,58,59,60,61]. The CTL response has been associated with the appearance of escape mutations in HIV and SIV infection. The mechanism of escape observed for individual mutations can vary. Mutations can affect epitope processing, stability of peptides, MHC:peptide complex stability, or TCR recognition of the MHC:peptide complex. Mudd *et al.* observed that Mamu-B*00801 macaques that controlled viral infection acquired few, if any, escape mutations in Vif and Nef epitopes, whereas macaques that progressed acquired several during the acute phase, suggesting that control may result from a immunologic pressure that prevents the appearance of escape mutations[62]. In contrast, Migueles *et al.* found that there was no difference between HLA-B*57^+CPs and HLA-B*57^+ ES in the frequency of escape mutations in Gag[45], and Bailey *et al.* found a high frequency of escape mutations in HLA-B*57-restricted epitopes present in virus amplified from ES plasma[24,46].

In this study, we sought to determine how ES maintain control of viral replication despite circulating escape mutant viruses in the plasma. We constructed a series of mutants that contained commonly observed HLA-B*57 restricted Gag escape mutations. While our study is limited by the fact that we did not study viral inhibition of autologous escape mutants isolated from each ES, the A146P and T242N/G248A mutations in the IW9 and TW10 epitopes are commonly seen in our cohort[24]. Mutations in KF11 are rare in Clade B HIV-1 isolates, but one patient was found to have the A163S mutation and we demonstrated that this was in fact an escape mutation in a prior study[24]. In agreement with other studies[47,48,49,50,51], we found that some of the escape mutants we generated were detrimental to viral fitness. While attenuating escape mutations may contribute to

elite control[63], viruses from CPs have been observed to have similar escape mutations, although compensatory mutations may partially restore viral fitness[64,65].

Klenerman and Zinkernagel demonstrated a limitation to the adaptive immune response: original antigenic sin[66]. In brief, when CTLs respond to an intracellular pathogen, any variant of the original pathogen elicits the activation of the original memory response, which is potentially less effective in the face of the new variant of the pathogen[67]. Allen *et al.*demonstrated in a cohort of HLA-A*11$^+$ individuals that the CTL response that recognizes escape variants was incapable of recognizing the original, un-mutated variants, as these CD8$^+$ T cells express unique Vβ segments[68]. New CTL responses have been shown to arise not only during the acute phase, but during chronic infection in HLA-A*02$^+$ patients[69]. Lichterfeld *et al.* have shown that HLA-B*27$^+$ individuals can develop a *de novo* response to the immunodominant KK10 L268M escape mutation during chronic infection[70]. In a previous study, HLA-B*57/58$^+$ children infected perinatally showed a remarkable ability to generate *de novo* CD8$^+$ T cell responses to escape mutations in the TW10 Gag epitope. Interestingly, there was little recognition of the wild type TW10 epitope in these children[71]. Another study found that CD8$^+$ T cells from both HLA-B*57$^+$ ES and HLA-B*57$^+$ viremic patients made responses to autologous TW10 escape variant peptides[72]. Furthermore, we previously have described CD8$^+$ T cell *de novo* responses to escape mutants in HLA-B*57$^+$ES[24,73].

While of all these studies examined IFN-γ responses, secretion of this cytokine is not a correlate of immunity in HIV infection[74,75]. Furthermore, discrepancies between IFN-γ ELISPOT assays and CD8$^+$ T cell-mediated killing of both SIV and HIV escape variants have been reported[76,77]. Therefore in order to determine whether protective de novo responses were present in ES, we looked at the ability of CD8$^+$ T cells to suppress replication of escape mutants. We used the suppression assay because Saez-Cirion and

colleagues have demonstrated that the ability of unstimulated primary $CD8^+$ T cells to inhibit viral replication correlates with elite control of HIV-1 infection[78,80], and we have recently confirmed this finding[79]. In a prior study, we demonstrated that $CD8^+$ T cells from an HLA-B*57 ES suppressed multiple rare autologous TW10 escape variants by a non-cross reactive *de novo* response[81]. In the current study, we demonstrated that this phenomenon is not limited to that one ES or to rare TW10 epitopes. $CD8^+$ T cells from multiple HLA-B*5703$^+$ ES were able to suppress the replication of virus containing common escape mutations in all three HLA-B*5703-restricted Gag epitopes. This is probably due to the development of $CD8^+$ T cells that produce perforin in response to wild type and escape variant peptides.

Interestingly, while the presence of residual intracellular concentrations of antiretroviral drugs prevented us from infecting $CD4^+$ T cells and performing the suppression assay with cells from HLA-B*57$^+$ CPs, we demonstrated *de novo* IFN-γ production when $CD8^+$ T cells of some CPs were stimulated with variant peptides. This is consistent with an earlier study which showed that HLA-B*57$^+$ CPs made IFN-γ responses to autologous TW10 variants that were as strong, if not stronger, than the responses made by HLA-B*57$^+$ ES[72]. Thus the elite control of viral replication is not solely due to the ability to recognize escape mutants. Rather, our work suggests that ES maintain control of viremia in spite of virologic escape in immunodominant epitopes because they develop protective $CD8^+$ T cell responses to the escape variants. In contrast, CPs generally do not develop protective CTL responses[18,21,39,53,78,79,80], and a study that compared HLA-B*57$^+$ ES to HLA-B*57$^+$ CPs found that proliferative CTL responses as well as perforin secretion in response to HIV antigens correlated strongly with elite suppression[21]. Interestingly, we show here that stimulation with wild type and escape variant peptides can induce perforin responses to both peptides in some CPs. Thus, it

may be possible to immunize subjects with both wild type and escape variant peptides in order to induce protective $CD8^+$ T cell responses that will prevent the emergence of common escape mutations. Taken together, it appears that ES $CD8^+$ T cells may develop effective CTL suppressive responses to escape variants; these responses in addition to the reduced fitness of the escape variants, may explain how ES maintain levels of viremia in the face of virologic escape.

CONCLUSION

In this study, we demonstrate the ability of $CD8^+$ T cells from ES to suppress replication of viruses harboring escape mutations in HLA-B*57-restricted Gag epitopes. The reduced fitness of these escape mutants may also contribute to elite control. Additionally, protective *de novo* $CD8^+$ T cell responses to both wild type and escape variant peptides could be generated in ES and some CPs by priming PBMCs with either peptide. Induction of $CD8^+$ T cells that could respond to wild type virus as well as common escape mutants would be advantageous for a CTL-based vaccine.

MATERIALS AND METHODS

Consent

All studies were approved by the Johns Hopkins Institutional Review Board. All patients and HIV negative donors provided written informed consent before participation in this study.

Construction of escape mutant viruses

Single-round infection by pseudotyped NL4-3 virus has been previously described[82]. In brief, *eGFP* was introduced in the*env* reading frame of pNL4-3, thus creating an *env* deficient pNL4-3 that allows for analysis of infected cells by flow cytometry. Individual point mutations were introduced by site directed mutagenesis (Agilent Technologies QuikChange II kit) and primers: A146P 5′

ggcaaatggtacatcagcccatatcacctagaactttaaatgc, I147L 5′ ggtacatcaggccctatcacctagaactttaaatgcatgg, A164S 5′ ggtaaaagtagtagaagagaagtctttcagcccagaagtaatacc, and T242N/G248 5′ cccttcaggaacaaatagcgtggatgacacataatccacc followed by 5′ ggaactactagtaaccttcaggaacaaatagcgtgg. A146P/A163S was made by sequential mutagenesis. A146P/A163S/T242N/G248A was made by insertion of the digestion product of T242N/G248A with SpeI and SbfI into the A146P/A163S plasmid. These plasmids were sequence confirmed and individually cotransfected with pCI containing the III-B *env* reading frame into HEK293T cells using Lipofectamine 2000. 48 hours after transfection, supernatant was collected and virus was isolated by ultracentrifugation at 50,000 × g through a 20% sucrose cushion for 2 h.

PBMC peptide stimulation

PBMCs were isolated from blood of ES, CP, and healthy donors by Ficoll gradient centrifugation. PBMCs were then cultured in RPMI 1640 supplemented with 10% FBS and 10 units/mL IL-2 in the presence of either 10 ug/mL total of TW10, KF11, and IW9 peptide (3 ug/mL each), or corresponding peptides containing escape mutations, for 7 days, with IL-2 supplemented every 48 h.

$CD8^+$ And $CD4^+$T cell isolation

PBMCs were isolated by Ficoll gradient centrifugation. $CD8^+$ T cells were isolated from PBMC using the Miltenyi Human CD8 Microbeads according to the manufacturer's instructions. $CD4^+$ T cells were isolated from either bulk PBMCs or CD8-depleted PBMCs using the Miltenyi Human $CD4^+$ T Cell Isolation Kit II according to the manufacturer's instructions. Purity of both cell types was routinely greater than 95% as determined by staining with CD3-Pacific Blue and CD8 APC or CD4 PerCP-Cy5.5 (BD).

Suppression

Freshly isolated $CD4^+$ T cells were spinoculated as described[83] at 1,200 × *g* for 2 h with one of seven NL4-3 pseudotyped viruses in 2.9×10^6 cells/tube. We typically used 50 to 100 ng p24 virus for 100,000 $CD4^+$ T cells which typically resulted in 2 to 10% GFP positive cells. Cells without virus were spinoculated as a negative control. After spinoclulation, $CD4^+$ T cells were washed and plated in a 96 well plate at 0.1×10^5 cells / well in RPMI 1640 supplemented with 10% FBS. Unstimulated, wild type stimulated, or Mutant stimulated $CD8^+$ T cells were immediately added to the spinoculated $CD4^+$ T cells, at specified ratios. Cells were cultured for 3 or 5 days before fixation and staining (CD3 Pacific Blue, CD8 APC, BD) and analysis by flow cytometry on a FACSCanto II (BD).

Intracellular cytokine analysis

0.5×10^6 unstimulated or stimulated PBMCs (from above) were restimulated with peptide (2 ug/mL), anti-CD28, and anti-CD49d in the presence of GolgiStop and GolgiPlug (BD) for 12 h. After staining with CD3 PE and CD8 APC-H7 (BD), cells were fixed and permeablized with Cytoperm/Cytofix Kit (BD). Cytokines were stained using IFN-γ PerCP-Cy5.5 (BD) and Perforin FITC (Cell Sciences). Stained cells were analyzed by flow cytometry on a FACSCanto II (BD).

Viral quantification

Concentrated virus was quantified by q-RTPCR. Viral RNA was isolated in triplicate with ZR Viral RNA Kit (Zymogen), and RT was performed using Superscript III First-Strand Synthesis Kit (Invitrogen) using $poly(dT)_{20}$ primers. VQA qPCR was performed as described[84]. In brief, Taqman Fast Advanced Master Mix (Invitrogen) was used with primers 5′ cagatgctgcatataagcagctg and 5′ tttttttttttttttttttttttttgaagcac, and run on a ViiA7 (AB).

Fitness assay

PBMCs were isolated from healthy donors by Ficoll gradient centrifugation and activated in RPMI 1640 supplemented with IL-2 (100 units/mL) and PHA (1 ug/mL) for 3 days. $CD4^+$ T cells were isolated (as above) and spinoculated (1,200 × g, 2 h) in a 96-well plate with 0.1×10^6 cells / well, with different concentrations of each virus as shown in Figure 1. 72 h after spinoculation, cells were fixed (3.3% formaldehyde) and the level of infection for each concentration of virus was analyzed by flow cytometry on a FACSCanto II (BD). GraphPad Prism was used to generate model curves. Relative fitness was determined as a percentage of the maximal infection of an individual virus relative to the wild type control.

FIGURES

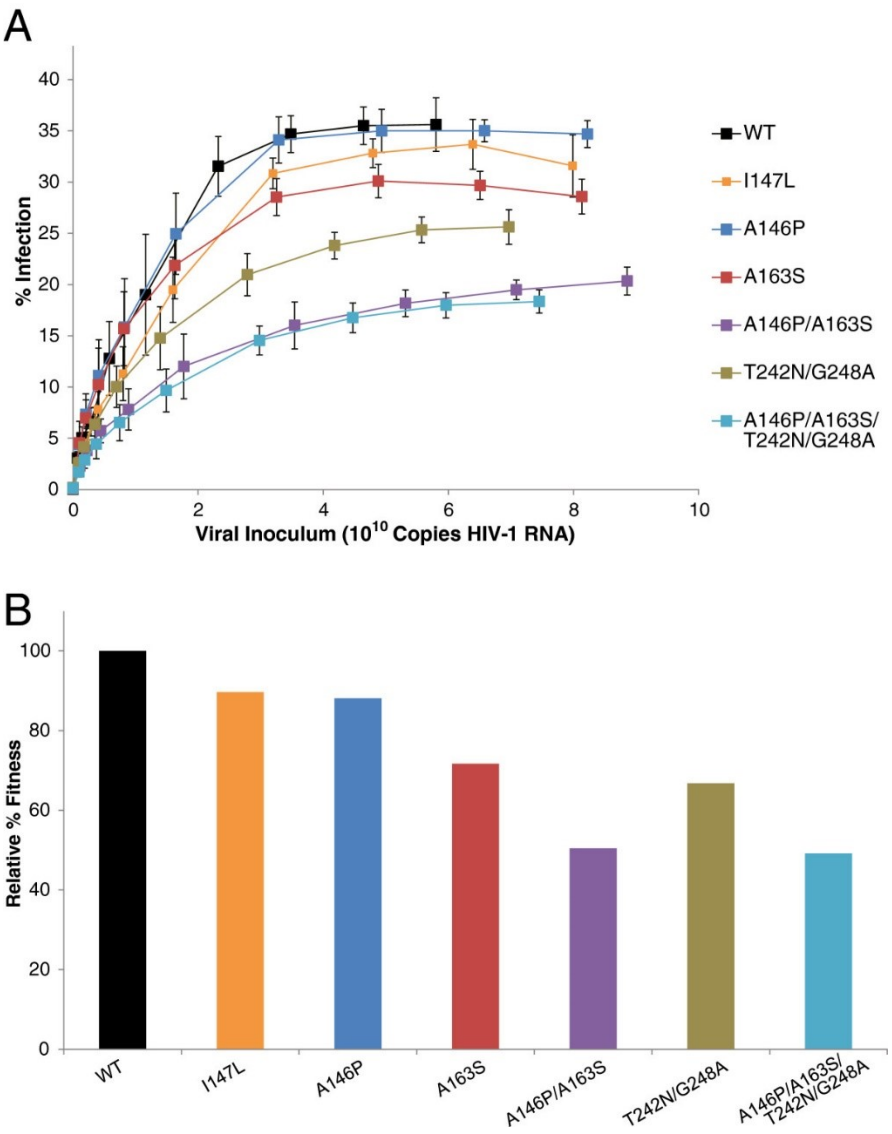

Figure 1

Fitness cost of canonical HLA-B*57 escape mutations. A. Average infection by seven NL4-3 escape variants in uninfected individuals. Viral inoculum was quantified by relative qPCR. 10^5 $CD4^+$ T cells were infected in a 96-well plate in triplicate. Infection was determined by GFP expression by flow cytometry. Wild type NL4-3 (black) showed the highest level of infection, while NL4-3 escape mutant variants showed reduced maximal infection (I147L, orange; A146P, navy; A163S, red; A146P/A163S, purple; T242N/G248A, brown; A146P/A163S/T242N/G248A, teal). Error bars represent SEM. n = 4. **B**. Maximal infection of each escape variant is compared relative to wild type NL4-3 virus.

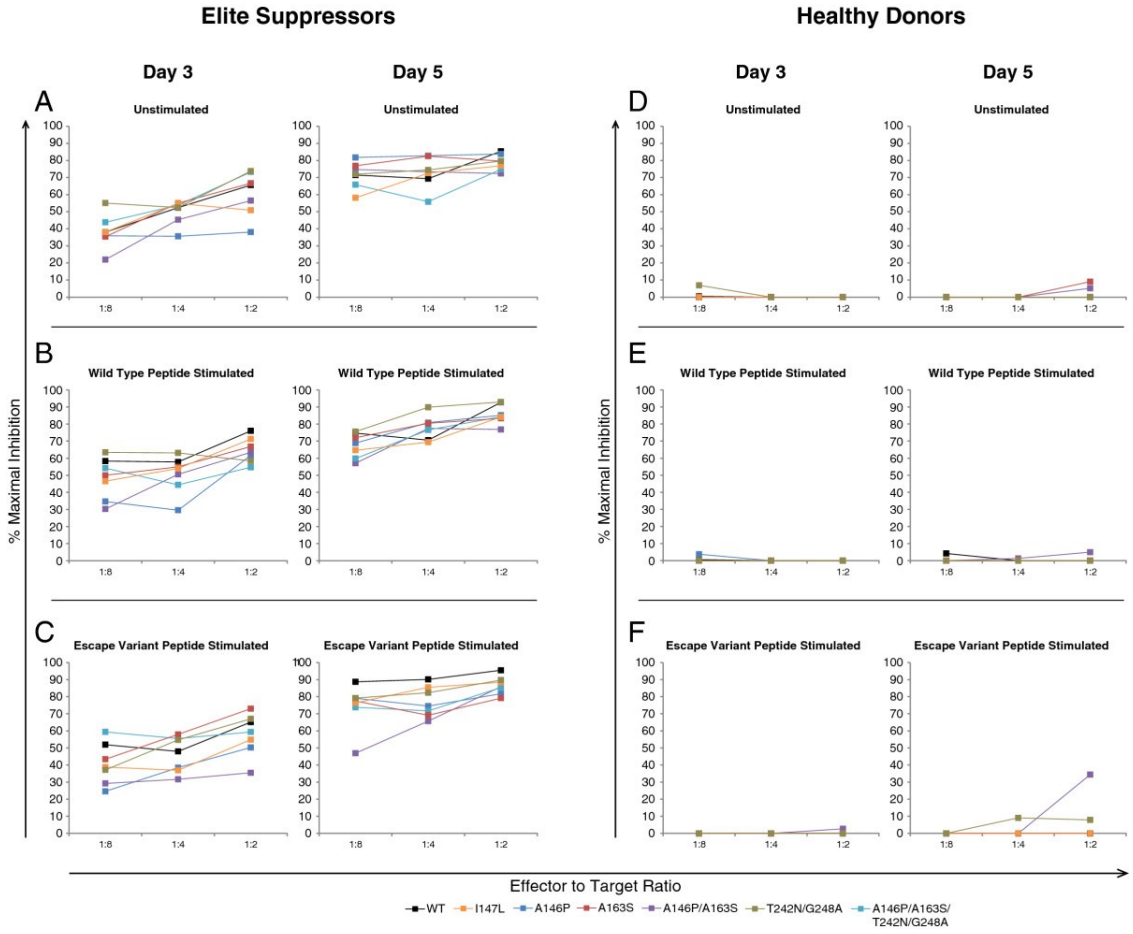

Figure 2

Suppression of replication of NL4-3 escape variant viruses. A-C: Unstimulated CD4$^+$ T cells from HLA-B*5703 positive ES were infected with one of seven NL4-3 variants (wild type, black; I147L, orange; A146P, navy; A163S, red; A146P/A163S, purple; T242N/G248A, brown; A146P/A163S/T242N/G248A, teal) and cultured with autologous CD8$^+$ T cells isolated from fresh PBMCs **(A)**or PBMCs stimulated with either wild type **(B)** or escape variant **(C)** HLA-B*57- Gag restricted Gag peptides for 7 days before isolation. CD8$^+$ T cells were co-cultured with infected CD4$^+$ T cells at three effector to target ratios. **D-F**: Unstimulated CD4$^+$ T cells from healthy donors were infected with one of the seven NL4-3 variants used above and co-cultured with CD8$^+$ T cells as was done with ES (**D**, unstimulated; **E**, wild type peptide stimulated; **F**, escape variant stimulated). Infection of CD4$^+$ T cells was quantified by flow cytometry on days 3 (left) and 5 (right) after infection by GFP expression. Median values are plotted. For ES, n = 7. For healthy donors, n = 5.

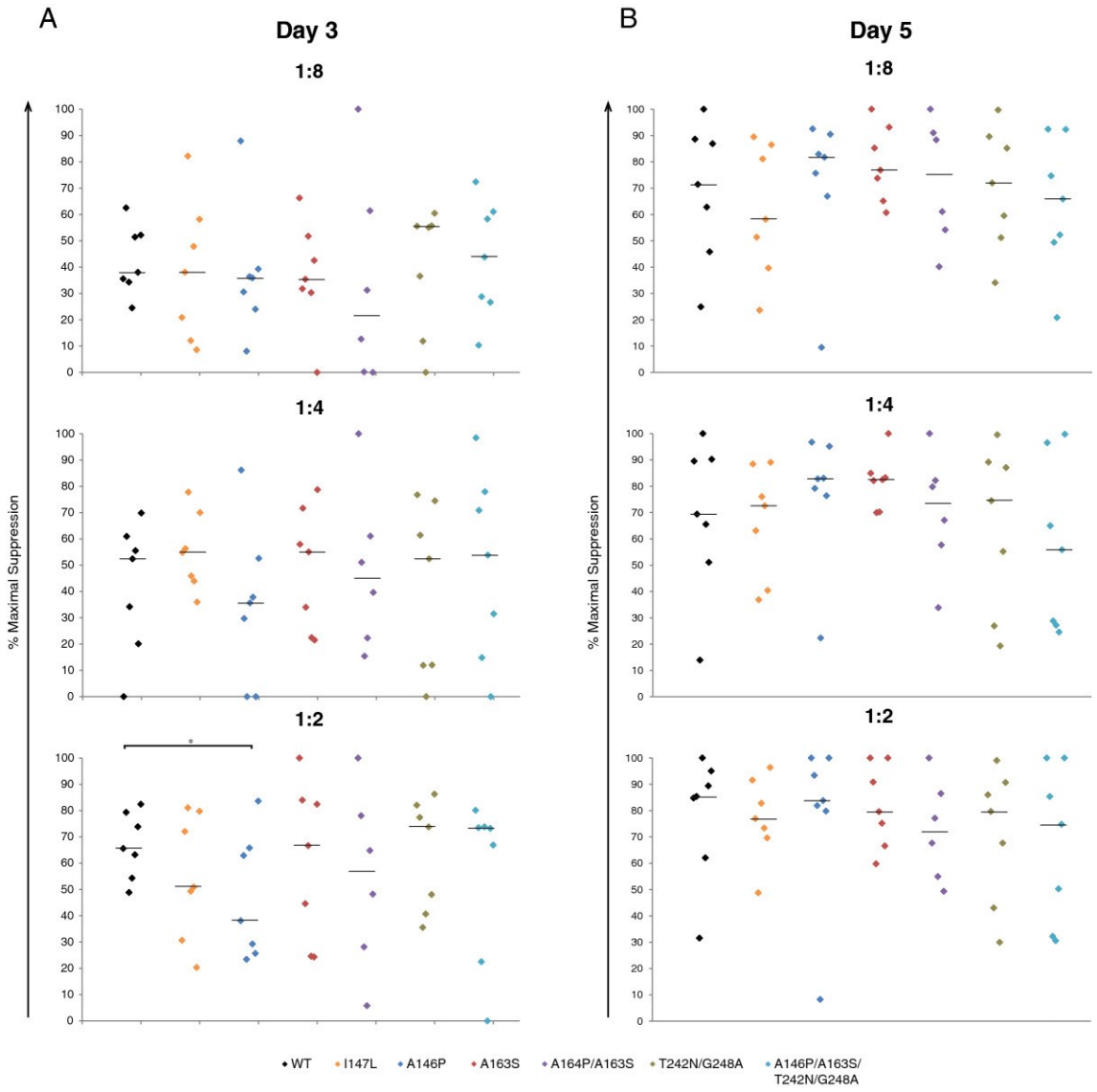

Figure 3

Individual suppression of NL4-3 escape variant viruses. Unstimulated $CD4^+$ T cells from HLA-B*5703$^+$ ES were infected with one of seven NL4-3 variants (wild type, black; I147L, orange; A146P, navy; A163S, red; A146P/A163S, purple; T242N/G248A, brown; A146P/A163S/T242N/G248A, teal) and cultured with autologous unstimulated $CD8^+$ cells at three effector to target ratios. **A** shows maximal suppression on day 3; **B** shows maximal suppression on day 5. Black horizontal bars indicate median. Asterisk indicates $P < 0.05$.

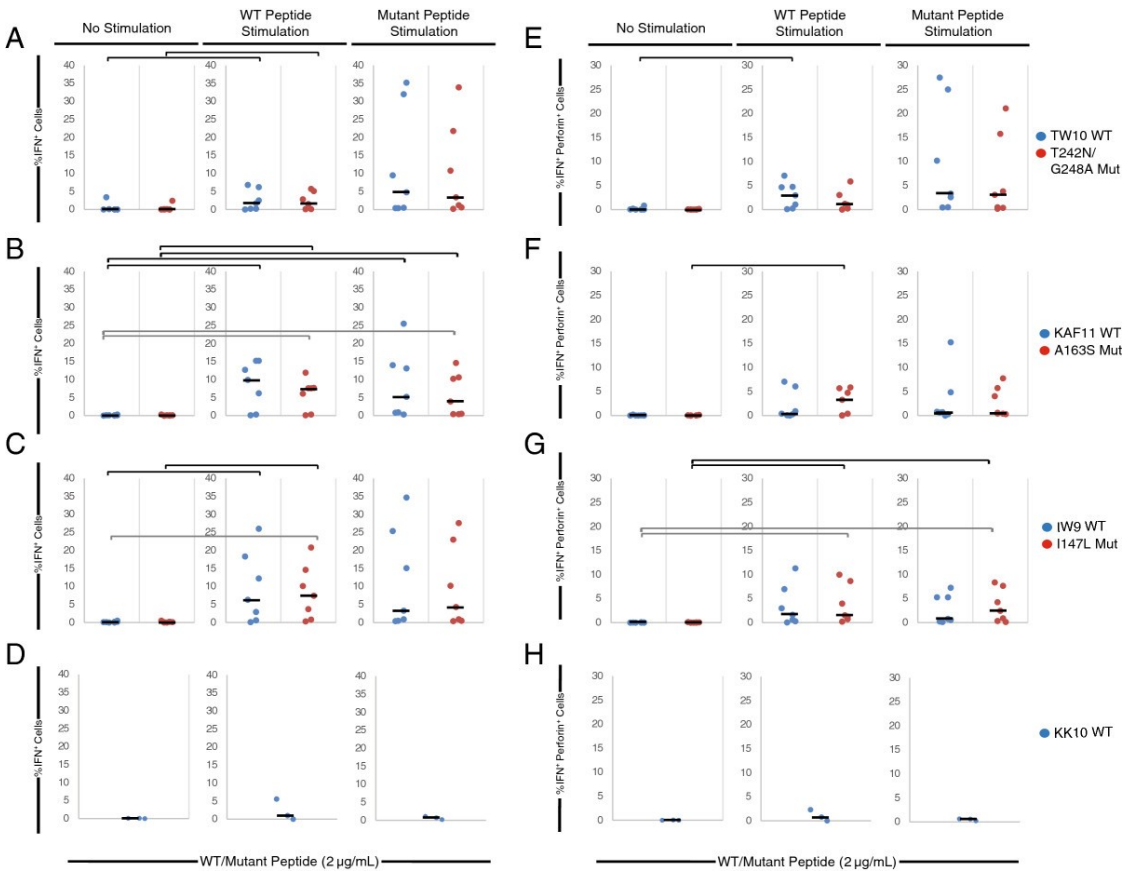

Figure 4

Intracellular cytokine staining of ES CD8 + T cells. A-D: CD8+ T cells of HLA-B*57 positive ES were either freshly isolated (left) or stimulated with either wild type (center) or escape variant (right) HLA-B*57-restricted Gag peptides for 7 days. Cells from each group underwent an overnight stimulation with individual peptides. Percentage of CD8+ T cells expressing IFN-γ when stimulated overnight with TW10 **(A)**, KF11 **(B)**, and IW9 **(C)** in blue, or the escape mutant variant peptide containing T242N/G248A **(A)**, A163S**(B)**, and I147L **(C)** mutations in red is shown. **E-H**: Percentage of CD8+ T cells expressing both IFN-γ and perforin after restimulation with TW10 **(E)**, KF11 **(F)**, and IW9 **(B)** in blue, or the escape mutant variant peptide containing T242N/G248A **(E)**, A163S **(F)**, and I147L**(G)** in red is shown. **D** and **H** show CD8+ T cells that express IFN-γ or co-express IFN-γ and perforin when PBMCs were stimulated overnight with Gag 263-272 (KK10, HLA-B*27+ peptide). Black horizontal bars indicate statistically significant difference ($P < 0.05$) between samples when stimulated overnight with the same variant peptide; gray horizontal bars indicate statistically significant difference ($P < 0.05$) between samples when stimulated overnight with opposite variant peptide.

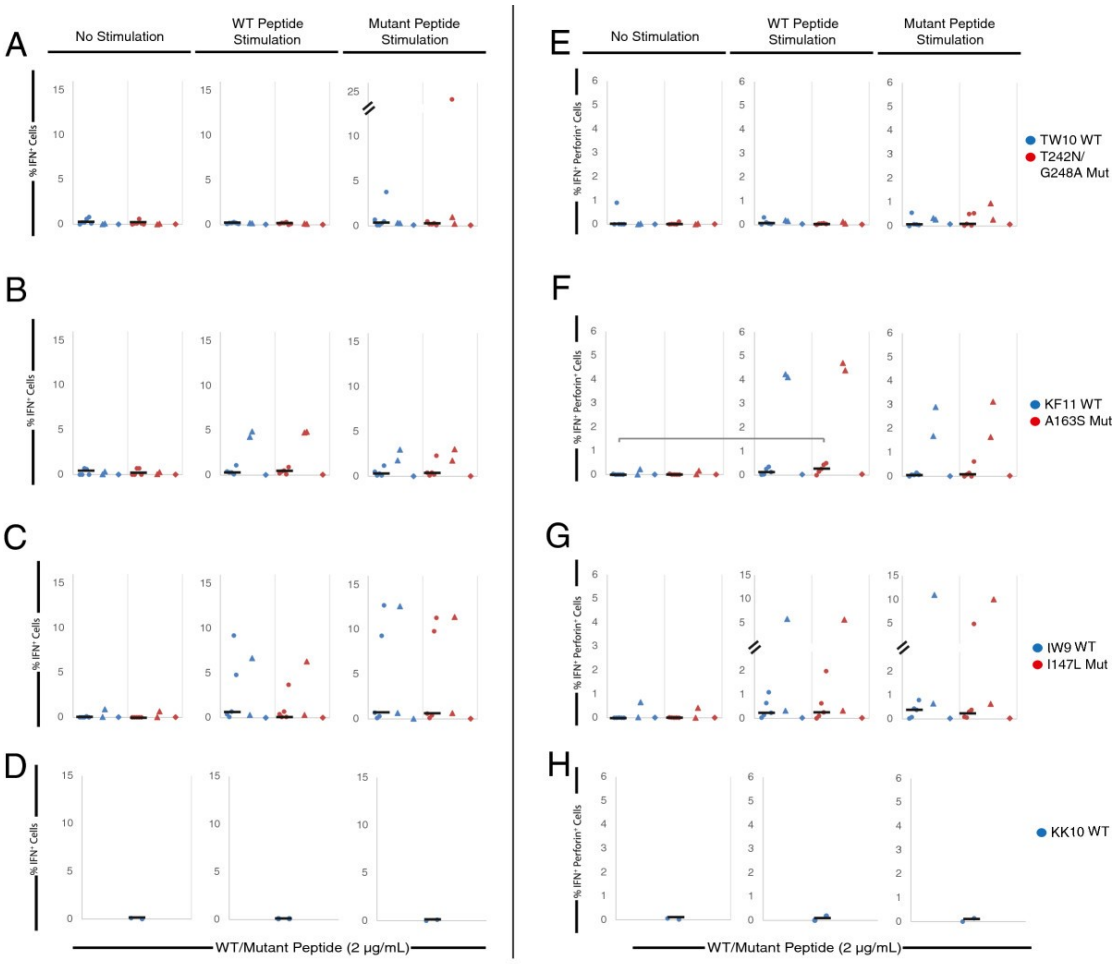

Figure 5

Intracellular cytokine staining of CP CD8⁺ T cells. A-D: CD8⁺ T cells of HLA-B*57 positive CP were either freshly isolated (left) or stimulated with either wild type (center) or escape variant (right) HLA-B*57-restricted Gag peptides for 7 days. CPs were either on suppressive HAART regiments with undetectable viral loads (circles), were on HAART regimens but recently had detectable levels of viremia (triangles), or were not on HAART and had high levels of viremia (diamond). Cells from each group underwent an overnight stimulation with individual peptides. Percentage of CD8⁺ T cells expressing IFN-γ when restimulated with TW10 **(A)**, KF11 **(B)**, and IW9 **(C)** in blue, or the escape mutant variant peptide containing T242N/G248A **(A)**, A163S **(B)**, and I147L **(C)** mutations in red is shown. **E-H**: Percentage of CD8⁺ T cells expressing both IFN-γ and perforin after overnight stimulation with TW10 **(E)**, KF11 **(F)**, and IW9 **(G)** in blue, or the escape mutant variant peptide containing T242N/G248A **(E)**, A163S **(F)**, and I147L **(G)** mutations in red is shown. **D** and **H** show CD8⁺ T cells that express IFN-γ or co-express IFN-γ and perforin when PBMCs were stimulated overnight with Gag 263-272 (KK10, HLA-B*27⁺ peptide). Black asterisks indicate statistically significant difference ($P < 0.05$) between samples when restimulated with the same variant peptide; gray asterisks indicates statistically significant difference ($P < 0.05$) between samples when restimulated with opposite variant peptide.

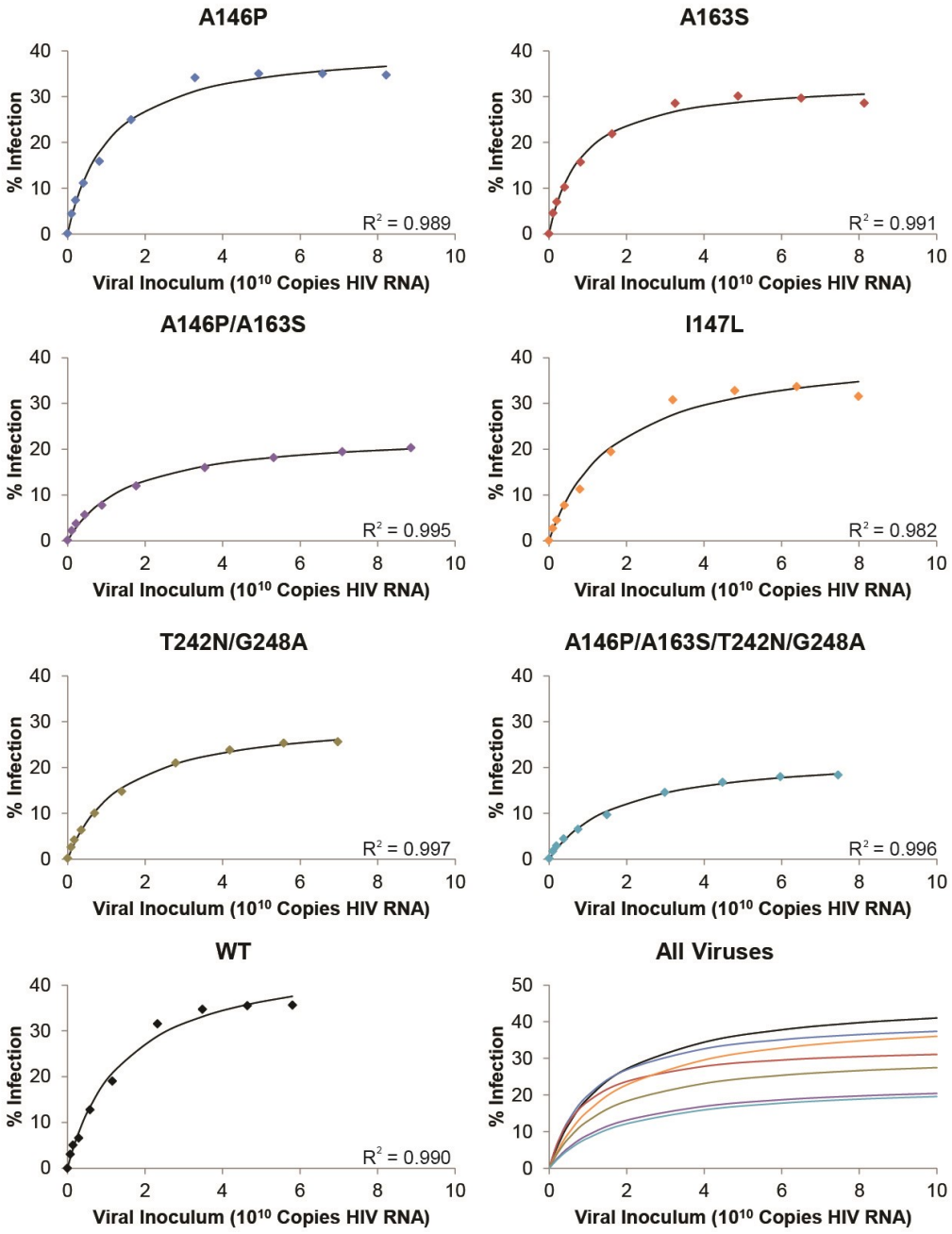

Figure S1

Theoretical nonlinear regression curves plotted with each escape variant. Data generated in fitness assay is shown here (wild type, black; I147L, orange; A146P, navy; A163S, red; A146P/A163S, purple; T242N/G248A, brown; A146P/A163S/T242N/G248A, teal). Black line depicts theoretical nonlinear regression curve generated by GraphPad. R^2 values for each theoretical curve is show on the bottom right of individual plots. Plot on the bottom right depicts all theoretical curves corresponding to NL4-3 variant color on the same plot.

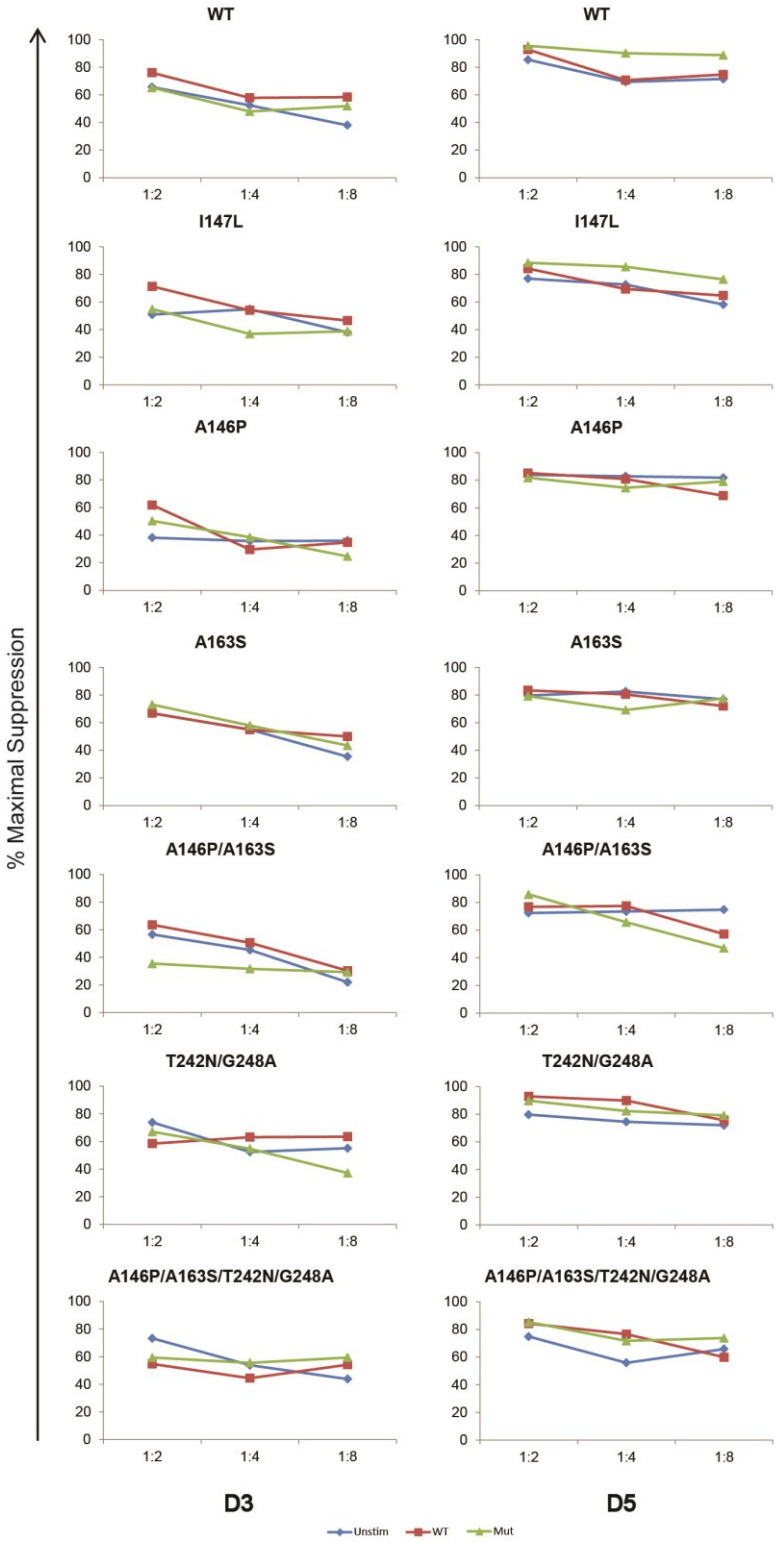

Figure S2

Comparison of stimulation status for suppressive function. Suppressive capacity of $CD8^+$ T cells, either unstimulated or stimulated with peptides corresponding to HLA-B*57 Gag epitope (WT) or escape mutant variant (Mutant), is compared for each of seven different escape mutant variant viruses used (wild type, black; I147L, orange; A146P, navy; A163S, red; A146P/A163S, purple; T242N/G248A, brown; A146P/A163S/T242N/G248A, teal). Suppression on day 3 (left) and day 5 (right) is shown. n=7.

CHAPTER 2: ELITE SUPPRESSORS HAVE LOW LEVELS OF CELL-ASSOCIATED HIV-1 MRNA THAT INCREASE UPON T CELL ACTIVATION

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*CPW, CKB, GML, and ARM contributed equally to this study

ABSTRACT

Objective:

Elite Controllers or Suppressors (ES) are patients who control HIV replication without antiretroviral therapy. In this study, we compared baseline and inducible HIV-1 mRNA levels in CD4+ T cells from ES and chronic progressors (CPs) receiving suppressive antiretroviral therapy.

Design/Methods:

We quantified basal levels of cell associated HIV-1 mRNA in CD4+ T cells isolated from CPs and ES. Additionally, we measured the fold upregulation of intracellular HIV-mRNA after stimulation of CD4+ T cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and quantified the amount of HIV-mRNA levels released into culture supernatant.

Results:

ES have significantly less cell associated HIV-mRNA per $5x10^6$ cells ($p = 0.003$); 8 of 10 CPs had quantifiable HIV-1 mRNA at baseline, whereas this was present in only 2 of 10 ES. Upon stimulation with PMA and ionomycin, 4 of 5 CPs and 7 of 9 ES showed increased cell associated HIV-mRNA. Interestingly, released HIV-1 mRNA could be detected in supernatants of CD4+ T cells stimulated with PMA/ionomycin from 5 of 8 ES.

Conclusion:

Our results demonstrate that while the baseline levels of cell associated HIV-1 mRNA are significantly lower in ES compared to CPs, stimulation of CD4+ T cells results in a comparable relative upregulation of viral transcription.

BACKGROUND

Elite controllers or suppressors (ES) are individuals infected with HIV that maintain undetectable viral loads without receiving antiretroviral therapy (ART). Prior studies have shown that replication-competent virus can be cultured from CD4+ T cells from some ES[7,8,9,10,23,25,85] and full genome sequence analysis of replication competent virus has not revealed the presence of mutations associated with attenuation[1,23]. Furthermore a recent study demonstrated that HIV-1 isolates from ES replicate vigorously and cause CD4+ T cell depletion in humanized mice[23]. Low levels of HIV-1 RNA can be detected in the plasma of most ES with ultrasensitive PCR assays[18,86,87,88], and sequence analysis has revealed evolution of plasma virus present in these patients[24,73,89,90,91]. These studies suggest that there is ongoing low-level replication in these patients, and yet in spite of this, ES maintain very small latent reservoirs as measured by total[10,15,92] and integrated DNA[93] as well as by the quantitative viral outgrowth assay[7] and the mouse viral outgrowth assay[94]. Despite the small reservoirs present in these patients, we hypothesized that ES would have relatively high levels of cell associated mRNA as a result of ongoing viral replication.

Hatano and colleagues have measured cell-associated in PBMCs isolated from ES[88] and have compared cell associated RNA present in CD4+ T cells of ES and CPs isolated from gut-associated lymphoid tissue[95]. These studies, however, did not specifically quantify HIV-1 mRNA which is a more direct indicator of HIV-1 transcription[96]. In the present study, we measure baseline and inducible levels of cell associated HIV-mRNA in peripheral CD4+ T cells of ES and CPs using a previously validated primer probe set[97]. These results deepen our understanding of the HIV-1 latent reservoir in ES.

RESULTS

Cell associated HIV-1 mRNA from unstimulated CD4+ T cells *ex vivo* was quantifiable in only 2 of 10 ES (median<10 copies/5M cells) as opposed to 8 of 10 CPs tested (median=92 copies/5M cells; p=0.03) (Figure 6a). Proviral HIV DNA was measured in 10 ES (median of 52 copies per 10^6 CD4+ T cells) and 10 CPs (median of 309 copies per 10^6 CD4+ T cells) to verify that our patients represent previously seen ES and CP cohorts (data not shown).

To determine whether transcription was induced with T cell activation, we compared cell associated HIV-1 mRNA at baseline and after maximal T cell activation. Treatment of CD4+ T cells with the T cell activation control of PMA plus ionomycin (PMA/I) increased cell associated HIV-1 mRNA levels in 7 of 9 ES and 4 of 5 CPs (Figure 6b) . The measured cell associated HIV-1 mRNA per 10^6 CD4+ T cells remained statistically higher for CPs (median=33,676 per 10^6 cells) than for ES (median=873 per 10^6 cells) (p=0.047). However, there was no statistical difference between fold increase of cell associated HIV-mRNA between ES (13.1-fold, interquartile range=12.1) and CPs (19.2-fold, interquartile range=13.4, p=0.22) per 10^6 stimulated cells. Interestingly, cell associated HIV-1 mRNA levels remained below the limit of detection in 2 ES after stimulation with PMA and ionomycin stimulation.

We also assessed the effect of T cell activation on viral release into culture supernatant. Upon stimulation of replicates of $5x10^6$ CD4+ T cells from ES with PMA and ionomycin, HIV-1 mRNA was detected in the culture supernatant from 5 of 8 ES (Figure 6c). 3 ES (ES6, ES9, and ES42) had undetectable HIV-1 mRNA measurements in culture supernatant even when a total of 10-$20x10^6$ CD4+ T cells were stimulated. In contrast, in ES22, ES23, and ES24 we detected HIV-1 mRNA in the culture supernatant from the majority of stimulated CD4+ T cell replicates. Interestingly in ES36 and ES43, HIV-1 mRNA was detected in the culture supernatant in only 1 of 5 stimulated replicates, with

3,012 and 5,116 copies of HIV-1 mRNA present in the positive wells. The frequency of detected released HIV-1 RNA suggests an 89% probability by Poisson distribution that the signal observed reflects a single cell releasing virus, and our HIV-1 mRNA measurements fit with our recent estimates of the burst size of an infected CD4+ T cell *in vitro*[98]. Thus of the $25x10^6$ cells stimulated, it appears that only 1 cell harbored an inducible virus in both of these patients. Interestingly, ES36 and ES43 also had undetectable cell associated HIV-1 mRNA in $5x10^6$ CD4+ T cells after stimulation with PMA and ionomycin. Taken together, these data suggest that there is significant heterogeneity in the inducible HIV-1 reservoir seen in ES which is consistent with results obtained from the viral outgrowth assay[7,23].

DISCUSSION/CONCLUSION

Control of HIV-1 without ART by ES can be considered a model of a functional cure of infection. We have previously shown that these patients harbor much lower frequencies of latently infected CD4+ T cells[7] than CPs on suppressive ART regimens[99], and in this study we further analyze the inducible reservoir in ES. We demonstrate that cell associated HIV-1 mRNA levels observed in ES CD4+ T cells at baseline were significantly lower than those observed in CPs. These results further characterize the nature the latent reservoir in ES patients recent studies that found that ES had low levels of HIV-1 RNA in peripheral blood and in the GALT[88,95]. However, despite the low baseline levels of HIV-1 mRNA, there was no significant difference in the fold increase of cell associated HIV-1 mRNA levels upon PMA and ionomycin stimulation in CD4+ T cells isolated from ES and CPs, suggesting a similar induction of transcription in response to PMA and ionomycin treatment in both patient populations. While we cannot determine if this increase in HIV-1 mRNA is due to transcription of defective or intact proviral sequences[100], the data are consistent with our ability to culture replication-competent virus from some ES.

There was significant heterogeneity in the amount of cell associated and released virus seen in CD4+ T cells from different ES. Interestingly, ES23 and ES24 had detectable HIV-1 mRNA at baseline and both patients had a relatively high frequency of CD4+ T cells that released virus into culture supernatant following T cell stimulation. In contrast, the frequency of CD4+ T cells that released virus into culture supernatant was much lower in other ES. However we were able to culture and characterize replication-competent virus from 2 such patients, ES9[9] and ES36[24] in prior studies. Further studies will be needed to determine whether virus that is releases into culture supernatant following CD4+ T cell activation is in fact replication-competent.

Studies have shown that virus in the plasma of ES evolves over time suggesting that there is ongoing viral replication[24,73,89,90,91]. In spite of that we still found very low levels of HIV-1 mRNA in peripheral CD4+ T cells in some of these patients. It is possible that the robust CTL response in these patients[101] is able to eliminate transcriptionally active CD4+ T cells in peripheral blood that may be producing viral proteins, while CD4+ T cells in anatomical compartments that exclude CTL are the true source of ongoing viral replication. Results from a recent study that found that CD8+ T cells are excluded from B cell follicles in ES monkeys may be consistent with this hypothesis[102].

In summary, we characterize baseline cell associated HIV-1 mRNA and show a robust upregulation following T cell stimulation in some ES. The results will have implications for patients who are subjected to curative procedures but still have residual low level HIV-1 reservoirs.

MATERIALS AND METHODS

Study Participants:

All studies were approved by the Johns Hopkins Institutional Review Board. All patients provided written informed consent before participation in this study. CPs are patients

with undetectable viral loads on suppressive ART regimens for at least 1 year. ES have maintained undetectable viral loads by standard commercial assays without ART.

Isolation of HIV-1 mRNA and DNA:

PBMCs were isolated from fresh blood samples by Ficoll gradient centrifugation. $CD4^+$ T Cells were isolated using Miltenyi $CD4^+$ T Cell Isolation Kit and cultured in RPMI1640 supplemented with 1% Pen/Strep and 10% FBS. $5x10^6$ cells in 2 mL of complete media were either cultured in the presence of DMSO (0.2%) or PMA (50 ng/mL) and Ionomycin (2 μM) for 24 hours. This stimulation resulted in greater than 90% of T cell activation as determined by CD69 expression. Supernatants were mixed with Trizol LS and cells were lysed using Trizol. Total RNA was isolated as described previously[96]. Human genomic DNA was isolated using Puregene kit (Qiagen) as per manufacturer's instructions.

Quantification of HIV-1 mRNA and DNA:

Total intracellular RNA was reverse transcribed using qScript from Quanta Biosciences as per manufacturer's instructions. Reverse transcribed HIV-1 mRNA was quantified as described previously[96] using a primer and probe set that anneals to a highly conserved region of the 3' end of HIV-1 mRNA[97]. The limit of quantification was set as the dilution point at which the Ct of the plasmid molecular standard replicates had an s.d. > 0.5. We determined that the limit of quantification for all intracellular HIV-mRNA transcripts was 10 copies[95]. HIV-1 proviral DNA levels were quantified by qPCR using ToughMix (Quanta Biosciences). Previously published primers that target a small region of Gag[103] were used to detect HIV-1 proviral DNA, and cellular input was quantified measuring RNaseP levels (ThermoFisher) of a human genome standard (Roche).

FIGURES

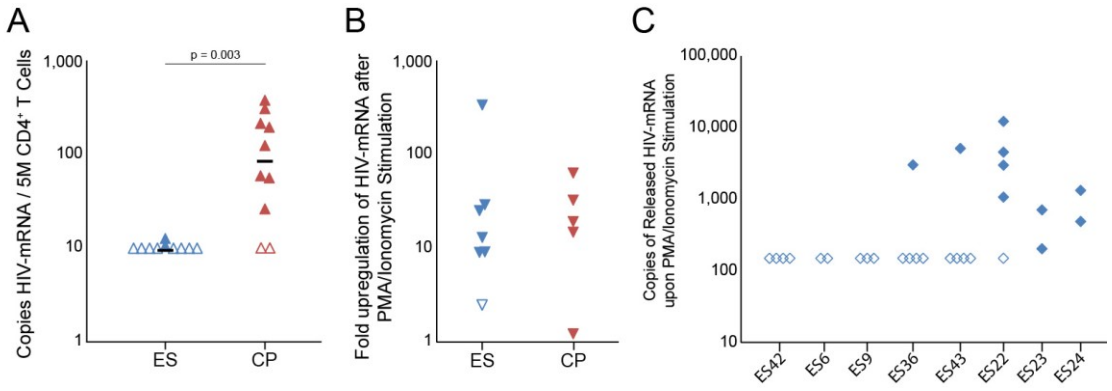

Figure 6

HIV-mRNA before and after PMA and ionomycin stimulation. **A** HIV-mRNA isolated from CD4+ T cells of ES (blue triangles) and CPs (red triangles) was quantified by qPCR. Samples below the limit of detection are represented by open triangles. The black horizontal bar represents the median value of HIV-mRNA for each population. HIV-mRNA levels observed were higher in CD4+ T cells of CPs than of ES ($p = 0.003$). **B** CD4+ T cells of ES (blue inverted triangles) and CPs (red inverted triangles) were stimulated with either DMSO or PMA and ionomycin for 24 hours and HIV-mRNA was isolated and quantified by qPCR. Fold upregulation upon PMA and ionomycin stimulation is plotted. An empty symbol indicates the limit of detection was used as the DMSO control value. No significance difference in fold upregulation was detected between the two populations ($p = 0.27$ with empty symbol, 0.24 without empty symbol). **C** HIV-mRNA release from CD4+ T cells upon PMA and ionomycin stimulation. Replicates of $5x10^6$ CD4+ T cells from ES were stimulated with PMA and ionomycin for 24 hours, and supernatant was collected for measurement of released HIV-mRNA. Open diamonds indicate replicates with supernatant values below the limit of detection. Single replicates from 2 ES (ES 36 and ES43) were positive, suggesting release of HIV-mRNA from individual cells.

SIGNIFICANCE AND FUTURE DIRECTIONS

The data presented here represent individual studies performed in a single lab. If I have learned anything in graduate school, I have learned to not believe any single study (or series of studies if done by the same lab) until the results are confirmed independently.

Nevertheless, the results discussed in this thesis suggest that some HIV-specific CD8+ T cells from ES are able to recognize both wild type and escape variant MHC-restricted epitopes. Without the ability to escape CTL control, viremia may be tempered. Activation and differentiation of naïve CD8+ T cells that express cross reactive T cell receptors may be critical targets for an effective therapeutic HIV vaccine.

While CTL activity is more robust in the ES population, this doesn't necessarily negate multiple factors being critical for the phenotype. Demonstrating that infected CD4+ T cells of ESs do not seem to have different levels of inducible intracellular HIV mRNA than CPs after maximal stimulation suggests that the resting state of latently infected CD4s is no different between the two populations. This supports the notion that more effective CTL control in CPs may lead to better control of viremia.

Understanding the *mechanism* by which ES control viremia may not be sufficient for developing a therapeutic vaccine. Cross-sectional studies cannot delineate between cause or consequence of the ES phenotype, i.e. whether any observed phenotype is causing the host to control viremia or is merely a byproduct of the host's control. It may be necessary to determine the *cause* of the ES phenotype in order to develop a therapeutic vaccine.

In that regard, it is best to study patients during seroconversion. This is very difficult to do, because a) ES are a very small proportion of the HIV-infected population and b) they will not necessarily present to a clinic during acute infection as most ES do not suffer

from acute retroviral syndrome. To circumvent this issue, Indian rhesus macaques are often used as ES models, though pathogenesis of SIV is often quite different than HIV; additionally, the rhesus macaque ES model may not truly recapitulate what is observed in humans.

Due to the enrichment of specific HLA molecules with the ES phenotype, studying seronegative donors that possess these protective alleles may shed some light on the cause of control. There is evidence to suggest that exposure to an antigen may induce an adaptive response against both the original antigen and HIV[104]. Screening healthy donors for HIV-specific CD8+ T cells could demonstrate that the cause of immunological control is a pre-existing HIV-specific memory response. As finding an individual who would become an ES is rather challenging, inducing an HIV-specific phenotype in a humanized mouse model followed by HIV challenge and monitoring HIV viral load levels and CD8+ T cell activity may provide useful information on ways to induce the ES phenotype in individuals who would otherwise progress.

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CURRICULUM VITAE

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Educational History

2011-2016	Johns Hopkins University School of Medicine Baltimore, MD Ph.D., Biochemical Cellular and Molecular Biology
2007-2011	Johns Hopkins University School of Arts and Sciences Baltimore, MD B.S. Molecular and Cellular Biology

Research Experience

Metcalf Pate KA, **Pohlmeyer CW** *et al*. A murine outgrowth assay to detect residual HIV-1 in patients with undetectable viral loads. J Infect Dis. Epub ahead of print (2015).

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Posters and Presentations

Pohlmeyer CW *et al*. Measurements of Viral Transcription in Elite Suppressors CD4+ T Cells. Themed discussion panel member. *CROI 2015.*

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Pohlmeyer CW et al. CD8+ T Cells from HLA-B*57 Elite Suppressors Effectively Suppress Replication of HIV-1 Escape Mutants. Poster. *CROI 2014*.